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Full Paper

Systematic adjustment of charge densities and size of polyglycerol amines reduces cytotoxic effects and enhances cellular uptake

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Excessive cationic charge density of polyplexes during cellular uptake is still a major hurdle in the field of non-viral gene delivery. The most efficient cationic vectors such as polyethylene imine (PEI) or polyamidoamine (PAMAM) can be highly toxic and may induce strong side effects due to their high cationic charge densities. Alternatives like polyethylene glycol (PEG) are used to 'shield' these charges and thus to reduce the cytotoxic effects known for PEI/PEG-core-shell architectures. In this study, we compared the ability of hyperbranched polyglycerol amines (hPG amines) with different amine densities and molecular weights as non-viral cationic vectors for DNA delivery. By adjusting the hydroxyl to amine group ratio on varying molecular weights, we were able to perform a systematic study on the cytotoxic effects caused by the effective charge density in correlation to size. We could demonstrate that carriers with moderate charge density have a higher potential for effective DNA delivery as compared to high/low charged ones independent of their size, but the final efficiency can be optimized by the molecular weight. We analyzed the physicochemical properties and cellular uptake capacity as well as the cytotoxicity and transfection efficiency of these new vector systems.

Introduction

The usage of cationic polymers as non-viral gene delivery vectors has been intensively studied over the last few decades.[1] Alternatively, viral gene delivery vectors have achieved the most efficient transfection rates for DNA delivery *in vivo* but also have serious tolerability issues.[2] In contrast, non-viral gene delivery vectors provide a number of promising advantages such as good biocompatibility and the potential for an easy scale-up as well as adjustability in size and charge. [3–5] The synergy between their multivalent binding effects based on different charge densities and sizes enables a broad range of biological applications in the field of gene therapy,[6] such as selective targeting or triggered release. However, one important challenge is the balance between transfection efficiency and cytotoxic effects.[7] Furthermore, cationic polymers have to fulfill several requirements including stabilization and protection of DNA from nucleolytic degradation, endosomal escape, and cytoplasmatic release. Recent studies concerning the correlation between size, charge, and dispersibility have demonstrated that cationic particles with a high surface reactivity are more toxic than larger hydrophobic or poorly dispersed particles.[8] It has been reported that nanoparticles with a size over 10 nm cannot be effectively cleared by the kidney as long as they are not biodegradable.[8]

Recently, a study on size and surface charge based on styrene derivates with a size of 50 nm and 100 nm revealed that the effective positive charge density dramatically influences the cellular uptake efficiency and the induction of cytotoxic effects.[9] In the case of cationic polymers, polyethylenimine (PEI) has remained the 'gold standard' since its discovery and use in gene delivery since 1995.[10] PEI is the most efficient polymer based transfection agent *in vitro* followed by PAMAM dendrimers.[11] Both polymers are still used as a positive control in many disease models but suffer from their high positive charge density. It is known that the remaining positive charge after condensation of DNA leads to apoptosis and necrosis of cells due to destabilization of the cellular membrane.[12,13] Therefore, lower molecular weight PEIs were intensively studied to avoid the remaining positive charge.[14] Until now, coupling polyethylene glycol (PEG) to the polymer's surface (PEGylation) or acetylation of the functional groups have been the major procedures for reducing the charge of highly positive DNA delivery vectors. This is known as 'shielding' or 'stealth' effect and has been intensively studied on PEI/PEG-derivates.[15] It could be shown that 50% of the PEGylated liposomes had an improved and longer circulation time in the blood than conventional liposomes.[16] Besides PEG, hyperbranched polyglycerol (hPG) is a promising candidate because of its similarly high biocompatibility, a globular structure with a high density of hydroxyl groups, biological inertness *in vivo*, tunable end group functionalities, and inertness to non-specific interactions with the biological environments.[17,18] hPG has a similar or even better biocompatibility profile than PEG with an adjustable MW

ranging from 4.2 kDa to 670 kDa.[19–21] *In vivo* studies on mice did not reveal any toxic side effects after intravenous injection of hPGs with a MW of up to 540 kDa and a total dose of up to 1 g kg^{-1}.[22] Recently, studies performed on hPG cores demonstrated a size-dependent endocytotic mechanism for cellular uptake of IDCC-labeled hPGs differing in molecular weight.[23] The hPG with a molecular weight of 475 kDa showed the best cellular uptake behaviour of these almost neutral polymer derivates.

In order to evaluate the potential of these polyvalent nanocarriers for gene delivery, we synthesized different hyperbranched polyglycerol amines (hPG amines), based on hPG cores with varying amine to hydroxyl group ratios and characterized them according to their physicochemical properties. Our analysis studied and compared the influence of the charge in correlation with the size of the polymer affecting their transfection efficiency and cytotoxicity. We could show that the best transfection efficiency and lowest toxicity was achieved by a molecular weight of 200 kDa.

Materials and methods

Materials

All starting materials and absolute solvents were commercially available and used without further purification. Glycidol was dried over CaH_2, distilled under reduced pressure, and stored under argon atmosphere at 4 °C. The addition of chemicals and the air and the moisture sensitive reaction were performed under inert gas atmosphere and the glassware was dried at a high temperature and flushed with argon, respectively, three times before usage. Dialysis was done in benzoylated cellulose dialysis tubes from Sigma-Aldrich (No. D-7884, width: 32nm, molecular weight cut-off ((MWCO) 1000 g mol^{-1}). The synthesis of the high molecular weight polyglycerol cores was similarly to the one performed in recent findings from D. E. Brooks group.[24] Furthermore, a scale-up reaction was investigated by fine-tuning the polymerization parameters.

General synthesis of hPG amines

General preparation of the initiator

In a three-necked 250 mL flask with a KPG -stirrer, the amount of 1,1,1-tris (hydroxymethyl)propan (TMP) (12.3 mmol, 1.65 g) was dried for 45 min at 95 °C. KOMe (3.6 mmol, 1.05 mL) was added to the reaction mixture under argon and kept overnight at 95 °C to evaporate the methanol. The mixture was stirred slowly.

Synthesis of polyglycerol cores up to 400 kDa

Tetrahydropyrane (THP) was added and the suspension was stirred for at least 30 min at 95°C to get all compounds into solution. The corresponding amount of glycidol was added with 14 mL/h. The reaction was kept overnight at 95°C, quenched with 100 mL methanol, and stirred for another 30-45 min until the reaction mixture was completely dissolved.

General reprocessing

A column was prepared with the ion exchanger (Amberlite IR120), activated with 5M HCl, and the reaction mixture was flashed at least eight times through the ion exchanger under gravity with increasing solvent (Methanol, 100 mL up to 1 L). The resulting solution was concentrated down and dialyzed for 5 d in methanol. Finally, the product was dried under HV for 4 d to get a colorless highly viscose liquid.

Yield: 55-67%, molecular weight distribution and PDI (GPC), ^{1}H-NMR (CD_3OD, 700 MHz): δ = 4.1-3.5 ppm (m, hPG-Backbone), ^{13}C-NMR (CD_3OD, 176 MHz): δ = 79.4 (hPG backbone, linear 1−3 units), 78.0 (hPG backbone, dendritic units), 72.2 (hPG backbone, linear 1−4 units), 70.7-70.4 (hPG backbone, linear/dendritic units), 69.2-68.9 (hPG backbone, linear 1−3/1−4 units), 62.6 (hPG backbone, terminal units) and 60.8 (hPG backbone, linear 1−3 units).

Table 1. Molecular weight determination by GPC of all synthesized hPG cores. The degree of branching (DB) was calculated according to an equation published by A. Sunder et al.[25] Further data are shown in the Supporting Information, Table S 1.

Sample	Mw / kDa	PDI	DB /%	THP/Glycidol (s/s)
60 kDa hPG	60	1.9	59	1:4
100 kDa hPG	99	1.9	60	1:2
200 kDa hPG	199	1.8	58	3:4
400 kDa hPG	400	2.0	59	10:9

The surface modifications on all polyglycerol cores were performed according to the published procedure.[26]

In short, hPG (1 g, 13.5 mmol OH groups) was dissolved in abs. pyridine (15 mL) and cooled down to 0 °C using an ice/sodium chloride bath. Mesylchloride (0.5 mL, 0.77 g, 6.75 mmol) was slowly added. The O-mesylpolyglycerol, gained after reprocessing, was dissolved in p.a. DMF, NaN_3 (4.39 g, 5 eq) was added and the reaction mixture was stirred overnight at 60 °C. After dialysis in methanol, the polyglycerol azide was reduced using PPh_3 (1.77 g, 6.75 mmol, 1 eq) in p.a. THF/H_2O (1:1). All obtained hPG amines are listed in the Supporting Information, Table S 1.

Synthesis of the labeled hPG amines

hPG amine (1 eq, 10 mg, 40.5 µmol) was dissolved in Milli-Q-Water, and 2-iminothiolane (3 eq, 16.7 mg, 121.5 µmol) was added to the solution. IDCC dye (3 eq) was added after 20 min of stirring. The mixture was stirred for another 2 h and reprocessed using a Sephadex G25 filter column. The residue was additionally dialyzed for two days to remove all of the remaining free dye. The dye per particle content was analyzed by UV/VIS measurements.

Physicochemical characterization

The molecular weight distribution was determined by means of GPC coupled to a multi angle laser light scattering (MALLS) - and a refractive index (RI) detector. The complete distribution (M_n, M_p, M_w, M_w/M_n) was obtained using highly diluted conditions (10 mg mL^{-1}, injected volume 20 µL) from the GPC that consisted of an Agilent 1100 solvent delivery system with isocratic pump, a manual injector, and an Agilent differential refractor meter. Three 30 cm columns (Polymer Laboratories PFgel Mixed C, 5 µm particle size) separated the aqueous polymer samples using water with 0.05% NaN_3 as the mobile phase at a flow rate of 1 $mLmin^{-1}$. The columns were operated at 25 °C with the detectors at 50 °C. The calibration was performed with a certified standard Pullulan (linear) from PSS. WinGPC Unity from PSS was used for data acquirement and interpretation.

NMR spectra were obtained using the following spectrometers: Bruker ECX 400 (400 MHz proton-resonance) and Bruker AVANCE 700 (700 MHz proton-resonance, solvent standards,

sample amount: ^{1}H-NMR: 5-15 mg, ^{13}C-NMR: 40-80 mg). Solvent calibration was performed according to the literature.[27] The degree of branching (DB) of all polymers was calculated from the inverse gated ^{13}C-NMR intensities using an equation from the literature.[25]

IR spectra were performed with a FT/IR-4100 LE 170VA (Jasco Cooperation, Groß-Umstadt, Germany). All samples were measured as highly viscose liquids using one drop of the concentrated pure compound directly onto the diamond and were carried out under air conditions at 25 °C. Before the background was measured and automatically subtracted, all the spectra were analyzed by the given software "Spectra Manager".

Dynamic light scattering (DLS) measurements were obtained using Malvern Zetasizer Nano ZS (Malvern Instruments GmbH, Herrenberg, Germany). All samples were measured at a constant scattering angle of 173° at 25 °C and freshly prepared just before measurement in PBS buffer (10 mM, pH 7.4, Fischer Bio Reagents) for the hydrodynamic size and zeta-potential measurements. The polyplexes were incubated for 30 min at room temperature after adding the correlated amount of hPG amine to the DNA solution. All measurements were repeated at least three times and the volume for the zeta potential was increased to 0.7 mL.

Cell viability assays

Cytotoxicity of the nanocarriers was evaluated through impedance measurement with an xCELLigence real-time cell analyzer (RTCA) from Roche Applied Science (Mannheim, Germany). In short, A549 cells (adenocarcinomic human alveolar basal epithelial cells) were cultured in Dulbecco's Modified Eagle Medium supplemented with 10% fetal bovine serum (BioChrom KG, Berlin, Germany), 100 U/mL penicillin, and 100 µg/mL streptomycin at 37 °C, 5 % CO2, and 99 % humidity and seeded in a 96-well E-plate (10.000 cells/well). The plate was placed in the RTCA and the impedance was measured at least every 15 minutes. The plate was removed from the RTCA after approximately 24 h and the nanocarriers were added in various concentrations. Doxorubicin and untreated cells served as a control. The plate was placed back in the RTCA and the real-time impedance measurement was continued for another 48 h. Analysis was performed with GraphPad Prism 5.01 software using end point data obtained from the RTCA. Growths curves were generated by the RTCA software 1.2.1.

Cellular uptake study

The cellular uptake of IDCC-labeled nanocarriers was monitored by confocal laser scanning microscopy (cLSM) and flow cytometry. For cLSM 50,000 A549 cells were seeded on 9 mm glass coverslips in each well of a 24-well plate and cultured for 24 h before adding the nanocarriers for 1, 4, and 24 hours, respectively. Cells were grown at 37 °C, 5% CO_2, and 99% humidity and maintained in Dulbecco's Modified Eagle Medium (D-MEM) supplemented with 10 % fetal bovine serum (BioChrom KG, Berlin, Germany), 100 U/mL penicillin, and 100 µg/mL streptomycin. For a qualitative analysis by cLSM, the cells were washed 3 times with PBS and fixed with 4% paraformaldehyde (PFA) for 20 min. Afterwards, cell nuclei were stained with 4',6-diamidino-2-phenylindole (DAPI). Cells were observed and imaged using a confocal laser scanning microscope (Leica DMI6000CSB stand) and Leica LAS AF software.

For flow cytometry, 150,000 A549 cells were seeded in each well of a 24-well plate and cultured for 24 h before adding the nanocarriers at 1, 4, and 24 hours, respectively. Cells were grown as described above. For a qualitative analysis by flow cytometry, the cells were washed 3 times with PBS and detached by trypsin. The detached cells were then transferred to a flow cytometry tube and centrifuged at 138 x g for 5 min. Supernatants were discarded and cells were fixed with 4% PFA for 10 min at 4 °C. The cells were centrifuged at 138 x g for 5 min to remove the PFA. The supernatants were discarded and the cells were re-suspended in 100 µl PBS supplemented with 1% FCS and 0.1% sodium azide. The fluorescence of the cells was measured in a FACScantor (Becton Dickinson, Heidelberg, Germany) and the analysis was done with Flowing Software 2.0.

Transfection efficiency

For the transfection efficiency study 25,000 HeLa cells were seeded in each well of a 24-well plate and cultured for 24 h in 500 µl of Roswell Park Memorial Institute medium (RPMI) (Gibco, Darmstadt, Germany) supplemented with 10% (FBS BioChrom KG, Berlin, Germany) and 1% non-essential amino acids (Gibco, Darmstadt, Germany) and at 37 °C, 5% CO_2, and 99% humidity. Nanocarriers were diluted in 50 µl Opti-MEM reduced serum medium and incubated for 10 min at RT. 1 µl of GFP-Plasmids pEGFP-C1 (1,28 µg ml^{-1}) was also diluted into Opti-MEM medium and incubated for 10 min. For complexation, diluted nanocarriers were added to the plasmid solution, incubated for 30 minutes and added to the respective wells. After 4 hours of incubation, the medium was replaced by 500 µl of supplemented RPMI and cells were grown for 24 hours. Before quantitative analysis by flow cytometry, fluorescence images of the cells were taken using a Zeiss Axio Observer Z1 epifluorescence microscope with the AxioVision software. For flow cytometry, cells were washed 3 times with PBS and detached by trypsin. The detached cells were transferred to a flow cytometry tube and centrifuged at 138 xg for 5 min. and resuspended in PBS supplemented with 1% FCS, 0.1% sodium azide, and 0,04% propidiumiodide. Fluorescence of the cells was measured in a FACScanto (Becton Dickinson, Heidelberg, Germany) and the analysis was done with Flowing Software 2.0.

Result and Discussion

The correlation between the charge density and the polymer's size plays a major role in cellular uptake behavior, transfection efficiency and cytotoxicity. A high positive charge density is necessary for good binding affinity with DNA, but excessive charge can cause necrosis and apoptosis in transfected cells.[14] The optimization of size and charge density may lead to a highly efficient and less toxic gene delivery vector. For a systematic analysis of those parameters, we designed and synthesized a library of hPG amines with varying charge densities. The PG90 14kDa ("PG-NH2") was resynthesized as an internal standard and served as a positive control for all experiments. This vector was the most efficient hPG amine candidate at the time.[28–30] To further improve this nanocarrier, we assigned the hPG amines into three categories with different amine loadings – low (5-15%), medium (30-50%), and high (80-100%) – on hPG cores

of three different sizes (60 kDa, 200 kDa and 400 kDa) (Supporting Information, Table S 1). Furthermore, a newly synthesis to scale up the polymerization of hPG cores was performed with batch sizes of 120 g according to the procedure described in the experimental section.

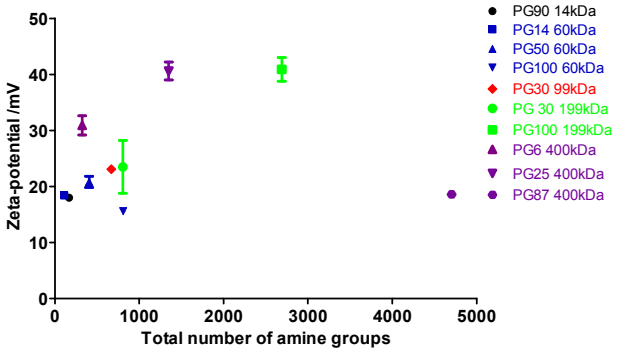

Figure 1: Zeta-potential measurements of all the synthesized hPG amines correlated to the total number of amine groups. The values are shown as mean±SD of at least triplicates.

First, we performed a preliminary cytotoxicity study consisting of all the synthesized hPG amines at three different concentrations (10 µg mL^{-1}, 100 µg mL^{-1}, and 1 mg mL^{-1}) to identify the best-suited candidates for the further *in vitro* transfection experiments. All the polymer derivates did not show any toxicity up to a concentration of 100 µg mL^{-1}. At 1 mg mL^{-1}, low- and medium -functionalized (5-50%) hPG amines only caused a minor or no decrease in the cells' viability; most of the highly -functionalized (80-100%) hPG amines significantly decreased the viability of the cells (Supporting Information, Figure S 1). These results can be explained by the fact that the enhanced number of hydroxyl groups on the same molecular weight led to reduced cytotoxicity.[31] Based on the first toxicity screening, another functionalized hPG with a core size of 100kDa with 30% amine loading (PG30 100kDa) was included in order to cover the wide molecular weight range from the first toxicity screening (Supporting Information, Table S 1). Furthermore, introducing PG30 100kDa made it easier to analyze the effect of the total amine groups correlated to size, charge and the polyvalent effect.

DLS measurements revealed that the hydrodynamic size of the hPG cores increased with molecular weights from 5 nm for the 14 kDa PG core up to 12 nm for the 400 kDa core and after modification of the hPG amines, a size increase of 3-9 nm was determined (Supporting Information, Table S 1). In general, the cationic charge increased with the total number of amine groups, which is correlated to the size and the degree of amine loading, from 15 mV to a maximum at 41 mV with a total number of up to app. 2500 amine groups per particle (Figure 1). However, a further increase of the total amine groups per core caused a decrease in the effective charge density as shown for PG87 400kDa with app. 4300 amine groups.

Based on the preliminary toxicity screening as well as on the zeta-potential measurements, we selected the five medium charged hPG amines with comparable amine densities and different core sizes for further experiments. Only this group showed reasonable cytotoxicity and a promising effective charge density to complex DNA. In addition, the use of hPG amines with a functionalization degree of less than 60% guaranteed that all amine groups were located on the surface.[25] Referring to the zeta-potential measurements (Figure 1), the selected five candidates entailed an increasing effective charge density from the lowest value (~18 mV) for PG90 14kDa up to the highest value (~41 mV) for PG25 400kDa. Furthermore, they all differed in their molecular weight, so that the effect of size and charge on cytotoxicity and transfection efficiency could be analyzed.

First, the polyplex agglomeration behavior was determined by DLS (Supporting Information, Figure S 2). These measurements at different nitrogen to phosphate (N/P) ratios up to N/P 100 revealed that the hydrodynamic size mainly depended on the added polymer concentration. The polyplex sizes seemed to have a maximum at N/P 3, which was two to three times higher than that of the pure hPG amines due to agglomeration effects (Figure S 2a). As long as the polymer concentration is not high enough to condense the entire added DNA, an agglomeration took place, and the polymer could be connected to more than one DNA strand. Starting from N/P 3 the size decreased again to a final hydrodynamic diameter similar to that of the pure hPG amines due to the higher influence of the polymer charge repulsion. The chance of agglomeration decreased which caused the DNA strands to separate and be surrounded by the hPG amines. In additional, all the polyplexes showed a highly positive zeta-potential which reflected their total number of amine groups similarly to the trend already measured with the pure polymer solutions (Figure 1 and Figure S 2 b). Conclusively, all tested compounds were able to complex the DNA at a low N/P ratio.

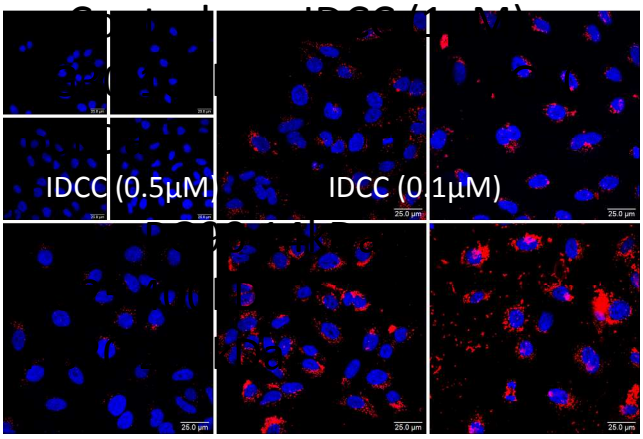

Figure 2. Images from the cellular uptake experiments taken after 24 h incubation of A549 cells with the different IDCC-labeled nanoparticles (red). Cell nuclei were stained with DAPI (blue). Untreated cells served as control as well as three different IDCC-dye concentrations.

For the cellular uptake experiments, all five polymer derivates were labeled with IDCC-dye according to the procedure described in the experimental section. Based on the preliminary *in vitro* cell viability results, non-toxic concentrations of the different polymer derivates were chosen for the uptake study and all following experiments. The cellular uptake of IDCC-labeled nanoparticles in A549 cells was monitored by a confocal laser scanning microscopy (cLSM) and flow cytometry for 1 h, 4 h,

and 24 h, respectively (Figure 2 and Supporting Information, Figure S 3). All polymer derivatives showed successful cellular uptake. The fluorescence images and flow cytometry results revealed that the effective charge density in correlation to the size influenced the cellular uptake efficiency; PG30 200kDa and PG25 400kDa showed a more efficient cellular uptake than PG90 14kDa, PG50 60kDa and PG30 100kDa. A higher amount of amine groups on the surface of the nanoparticle improved the effective charge density as well as the polyvalent effect. Hence, both factors increased the interaction of the polymer with the cellular membrane and resulted in an improved cellular uptake (Supporting Information, Figure S 4). The results are consistent with the reported trend.[23]

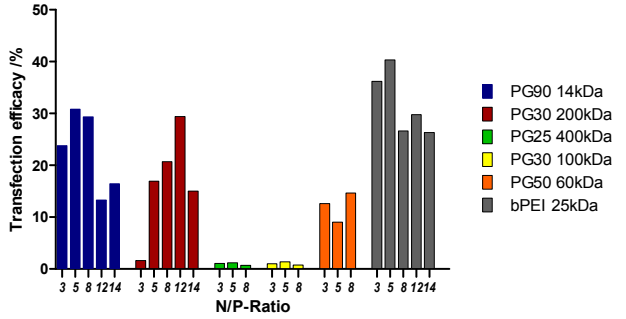

Figure 3. Transfection efficiency study with HeLa cells. The percentage of GFP expressing cells and therefore transfected cells relative to all cells was distinguished 24 hours after GFP-plasmid transfection. Each polymer was tested at different N/P ratios. 1.28 µg mL^{-1} plasmid DNA was used for complexation. The N/Ps 3, 5 and 8 were tested in the first screening, which was followed by a second screening for PG90 14kDa, bPEI 25kDa and PG30 200kDa with N/Ps 12 and 14.

Subsequently, two transfection studies to identify candidates with sufficient transfection efficiency were performed. Thereby, PG90 14kDa and bPEI 25kDa served as positive controls, since PEI is the gold standard for transfection studies. In a first screening at three different N/P ratios (3, 5 and 8, Figure 3)), PG30 200 kDa displayed good transfection efficiency followed by PG50 60kDa. Interestingly, PG25 400kDa with the highest effective charge density and the largest size was not able to transfect the DNA efficiently. Relying on the cellular uptake results, we expected higher transfection efficiency for this candidate. However, at N/P 8 PG30 200kDa demonstrated the best transfection efficiency comparable to PG90 14kDa. The high effective charge density of PG25 400kDa led to strong polyplex formation, which could have been a reason for the ineffective release of the DNA into the cytoplasm, since we could exclude the size effect due to good cellular uptake results. Only PG30 200kDa revealed promising results and a second transfection study at higher N/P 12 and 14 was performed (Figure 3). The results indicate that bPEI 25kDa remains the better transfection agent over the complete analyzed range, but at N/P 12 PG30 200kDa shows the best transfection results, which is comparable to bPEI 25kDa.

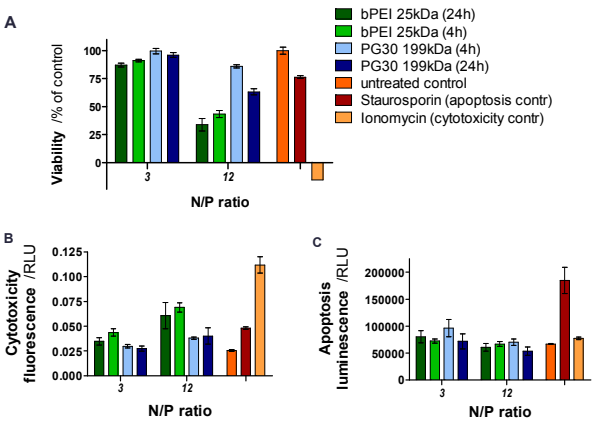

Figure 4. Using the promega kit (ApoTox-Glo Triples Assay), the cytotoxic effects were studied in detail; a) cell viability, b) cytotoxicity and c) apoptosis with the internal positive standards ionomycin (cytotoxicity) and staurosporin (apotosis).

Furthermore, we investigated and compared in detail the type and strength of cytotoxicity caused by PG30 200kDa to the gold standard bPEI 25kDa at N/P 3 and 12 (Figure 4) to confirm our hypothesis that an optimization of effective charge density in correlation with the size led to reduced cytotoxicity and enhanced transfection efficiency.

Using the promega ApoTox-Glo™ Triplex Assay Kit, three different parameters reflecting different cytotoxic effects were studied, (a) cell viability, (b) cytotoxicity and (c) apoptosis. Two internal positive controls were used in the test: ionomycin (100 µM) as an inducer of necrosis, and staurosporin (10 µM) as an inducer of apoptosis. Neither PEI 25kDa nor PG30 200kDa showed any serious toxic effects at N/P 3. At N/P 12 bPEI 25kDa demonstrated significantly higher toxicity and caused a higher decrease in cell viability than PG30 200kDa after 4h as well as 24h. No significant induction of apoptosis was observed for bPEI 25kDa or PG30 200kDa. Images taken by a Zeiss Axio Observer Z1 microscope revealed a higher amount of dead cells (round shape) in the bPEI 25kDa treated cultures compared to PG30 200kDa treated cultures (Figure 5). Conclusively, bPEI 25kDa seemed to be more toxic than PG30 200kDa, but both compounds induced necrotic effects.

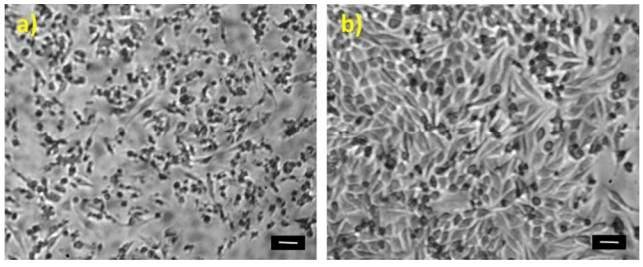

Figure 5. Toxic effects shown in HeLa cells incubated with a) bPEI 25kDa and b) PG30 200kDa at N/P 12 after 24h. Dead cells can be identified by the round shaped black dots and the scale of the white bar is 50 µm.

Conclusions

In conclusion, the effective charge density in correlation with the size of the polymer derivative plays a crucial role for biological applications. A high amine density of 90-100% independent of

the molecular weight causes significant cytotoxic effects. In contrast, a polymer with a low amine density of less than 20% has no effect. Consequently, neither polymers with a high nor a low amine density are suitable for cellular uptake and transfection.

Optimizing the amine groups per particle in correlation with the size showed that the cellular uptake was improved with increasing effective charge density and, as a result have a better ability to deliver DNA. In contrast to the pure hPGs, where a 475 kDa sized polymer showed the best cellular uptake.[23] For hPG amines, PG25 400kDa was less effective than PG30 200kDa. PG25 400kDa suffered from the high amine group density and showed agglomeration effects over time during biological testing, which reduced the efficiency dramatically. PG30 200kDa with an optimized effective charge density, did indeed demonstrate comparable cellular uptake and good transfection efficiency as well as a lower toxicity compared to the gold standard bPEI 25kDa. Altogether, we were able to decrease the cytotoxic effects and improve the cellular uptake by fine-tuning the effective charge densities affected by the size on the hPG amines. Further optimization on the amine density or introducing of targeting ligands using a PG 200kDa core will be necessary to design a highly efficient transfection agent.

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Notes

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† Electronic Supplementary Information (ESI) available: [details of any supplementary information available should be included here]. See DOI: 10.1039/b000000x/s

††All cell culture experiments were performed in accordance with the German genetic engineering law and German biosafety guidelines in an approved biosafety level 1 laboratory.

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